A Simple Method for the Quantitative Isolation and Resolution of Unesterified Fatty Acids from Fats and Oils

The unesterified fatty acids (UFA) present in fats and oils are isolated cleanly and without the formation of detectable artifacts due to saponification. The lipid (≤ 0.5 g) dissolved in 5 ml of n-hexane is passed over a 300-mg bed of Celite impregnated with saturated, aqueous Na_3PO_4 so that the solution passes through the bed in 3–4 min. After the bed has been washed, the UFA are freed from their salts by pumping of HCl vapor over the bed. The acids are eluted with CH_2Cl_2 and subsequently separated underivatized by gas liquid chromatography. The C_{10} through $C_{18:3}$ acids are separated in < 15 min. Recovery of the C_{10}–$C_{18:0}$ and $C_{18:1}$, $C_{18:2}$ and $C_{18:3}$ acids added to fatty acid-free fats and oils in several concentrations was nearly 100%

In connection with an anticipated study of the effects of γ irradiation on the lipids of chicken, a method for the quantitative isolation and resolution of the individual unesterified fatty acids (UFA) was sought. Although many methods are described in the literature for quantitating unesterified fatty acids, relatively few of these (1–4) are also applicable to the examination of the individual UFA in fats, oils and high lipid foods. The major problem with most of the methods is their potential for generating UFA as artifacts due to a relatively long contact time between the strong alkali used in the isolation procedure and the glycerides in the sample. As a consequence, we have developed a method for the quantitative extraction of UFA from fats and oils that is rapid and relatively simple, and minimizes the formation of artifacts due to saponification. The resolution of the individual fatty acids present in the extract without derivatization is also described. A preliminary report on the method has been presented (5).

EXPERIMENTAL PROCEDURES

Scope of method. The lipid dissolved in hexane is passed rapidly over a small bed of aqueous trisodium phosphate on Celite. The extracted acids are liberated from their salts by hydrochloric acid vapor, eluted and separated as free acids by gas liquid chromatography (GLC).

Purification of Celite. Celite 545 (Fisher Scientific Co., King of Prussia, Pennsylvania) was checked for suitability by subjecting it to the proposed procedure. If unsatisfactory it was purified by pumping the vapor above concentrated HCl over the Celite for a few minutes followed by its extraction in a Soxhlet extractor with CH_2Cl_2 for 4 hr and drying. Two of three lots of Celite examined were satisfactory for use without purification.

Preparation of Celite-trisodium phosphate. This extraction medium was prepared by grinding 2.5 parts of purified Celite with one part of a saturated aqueous solution of crystalline $Na_3PO_4 \cdot 12H_2O$ (pH~12) (J.T. Baker, Phillisburg, New Jersey) in a mortar. The mixture was stored protected from air.

Purification of n-hexane. The solvent was purified by passing 0.5 l over a 20 g bed of alumina (Alcoa F-20, A.H. Thomas Co., Philadelphia, Pennsylvania) discarding the first 50 ml. The alumina had been purified previously by washing one part with 2.5 parts high quality 2-propanol and then drying it at 130 C for 3–4 hr.

Preparation of fatty acid-free fats and oils. The lipid (1 g) in 10 ml of n-hexane was passed over a 4-g bed of acidic alumina (Fisher) which had been partially deactivated by the addition of 8% water. The sides of the chromatography tube (1.7 × 13 cm) were washed with 5 ml of CH_2Cl_2. Then the bed was eluted with 20 ml of CH_2Cl_2. The total effluent was evaporated at 40–45 C under a stream of nitrogen until the residue was constant in weight. The oils and fats subjected to this procedure were recovered (≥ 97%) and were free of unesterified fatty acids as determined by our procedure.

Isolation of UFA from fats and oils. A large volume Pasteur pipette (Fisher #13-678-8) was plugged with a 4-mm glass bead. Sand (ASTM grade) was added to just fill the tapered portion, then 300 mg of the Celite-Na_3PO_4 medium was added, settled by tapping, and tamped lightly. Hexane (5 ml) was pipetted into the column and the flow rate established. The column was considered potentially satisfactory if the last of the hexane entered the bed within 1.75–2.25 min. If the flow rate was faster the bed was tamped tighter and the flow rate reestablished; if slower, a new column was made. With experience columns could be made that had the desired flow rate with only minor deviation.

A 5-ml aliquot of a ≤ 10% solution of the lipid in hexane was passed over the bed, and the flow rate was noted. The last of the solution should enter the bed in 3–4 min. If slower than this the isolation should be repeated (see discussion). The sides of the tube were washed immediately with two one-ml portions of hexane. Then the tube was filled with hexane. After draining the tube, excess hexane was removed by application of pressure or vacuum. About a 0.5-cm layer of sand was added and the bed was tamped tightly. The acids were liberated from their salts by passing the vapor above concentrated HCl over the bed (Fig. 1) until the emerging vapor was acid to litmus (~30 sec). Gassing was then continued for an additional 30 sec. The bed was eluted with 1.5 ml of CH_2Cl_2 and the effluent was collected in a one-ml autosampler vial. The solvent was removed at room temperature under a N_2 stream. The residue was dissolved in a suitable volume of carbon disulfide and the vial sealed immediately with a Viton septum. The solution was let stand 15–30 min to equilibrate prior to analysis by GLC.

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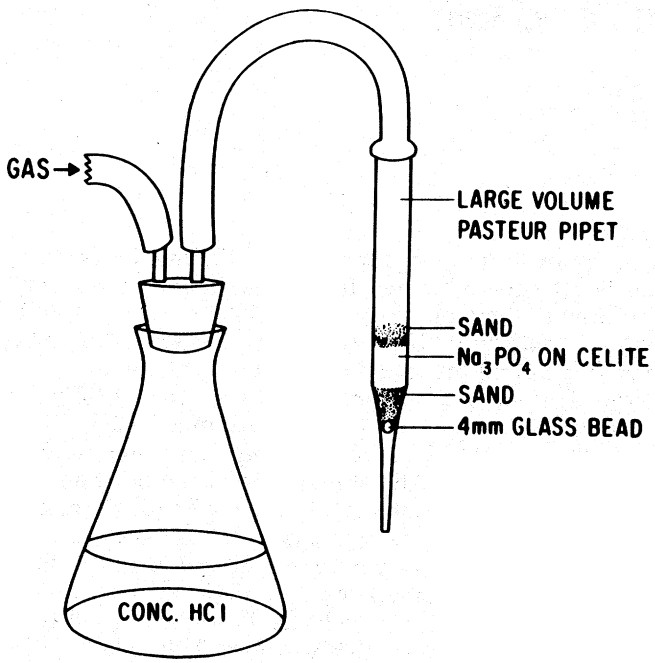

FIG. 1. Set-up for neutralizing Na_3PO_4 bed with HCl vapor.

GLC. The UFA were separated on a 6′ × 1/8″ glass column packed with 15% stabilized DEGS on 100–120 mesh chromosorb Z (Analabs, North Haven, Connecticut). The detector was flame ionization; the carrier gas was helium exiting from the column at 36 ml/min; injector and detector temperature were 250 C; column temperature was maintained at 195 C.

RESULTS AND DISCUSSION

Recovery of added fatty acids. The procedure was evaluated for its accuracy and precision by spiking fatty acid-free fats and oils with the common fatty acids and checking recoveries relative to the untreated standards. Recoveries were calculated from peak height measurements taking the average of at least five injections of both the standard and the isolated mixtures. Fatty acid-free sunflower, soybean, and coconut oils and lard and tallow were used as carriers in the recovery studies. Spikings were made at four different levels (all of which were multiples of the lowest level) into a 5–10% solution of the carrier. Recoveries are given in Table 1.

The acids studied were recovered in very good yield and with satisfactory precision. The lowest amount of spike designated in Table 1 was the least amount that afforded quantitative recovery, half of that amount giving erratic recoveries. Amounts larger than the largest amount listed were not studied. Acids lower than C_{10} were not investigated. It is expected that these would be recovered less than quantitatively due to partitioning and possibly also to losses incurred during evaporation of the CH_2cl_2.

Artifact studies. To establish to what extent, if any, saponification was occurring during passage of the lipid solution over supported Na_3PO_4, effluents from the first pass were cycled over another bed. No fatty acids could be detected in the residue from the second pass even when the sensitivity of the instrment was higher and even when the residue was dissolved in smaller

TABLE 1

Recovery of Fatty Acids Added to Fatty Acid-Free Fats and Oils

Fatty acid C No.	Range added (μg)	Recovery[a] % ± SD
10	8 - 64	99.1 ± 1.4
12	13 - 104	97.8 ± 3.4
14	13 - 104	96.2 ± 3.6
16	17 - 136	98.4 ± 4.5
18:0	19 - 152	98.2 ± 3.0
18:1	9 - 72	100.2 ± 4.1
18:2	10 - 80	97.9 ± 3.7
18:3	10 - 80	97.1 ± 3.4

[a]Average of recoveries of 11 spikings.

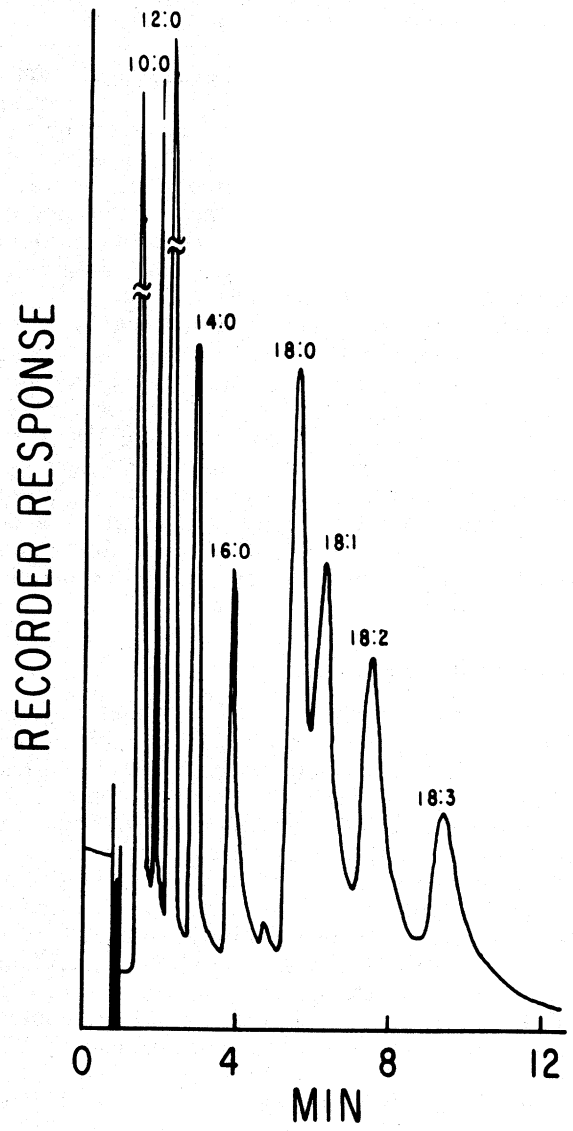

FIG. 2. Gas liquid chromatogram of standard fatty acid mixture. Column = 15% stabilized DEGS on chromosorb Z. Isothermal at 195 C.

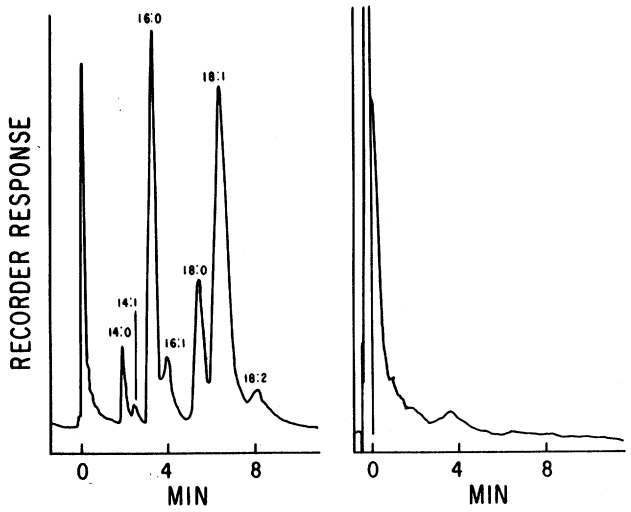

FIG. 3. a, Unesterified fatty acids isolated from 0.5 g of edible tallow. Injection is 1% of total residue. Attenuation = 128. b, Residue from second pass of 0.5 g of edible tallow. Injection is 1% of total residue. Attenuation = 8.

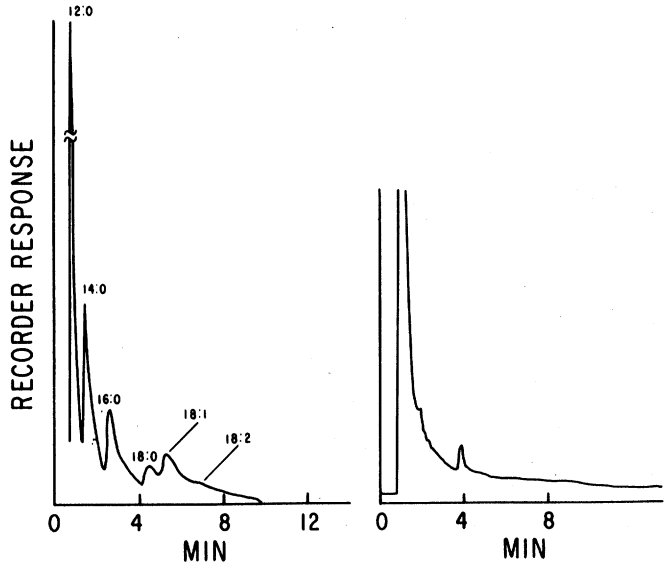

FIG. 4. a, Unesterified fatty acids isolated from 0.25 g of refined coconut fat. Injection is 1% of total residue. Attenuation = 16. b, Residue from second pass of 0.25 g of refined coconut fat. Injection is 2% of total residue. Attenuation = 2.

volumes of CS_2 and/or larger volumes were injected into the instrument. However, this was only true when the specified flow rates were strictly adhered to. When flow rates were slower, i.e., > 4 min/5 ml, some evidence for slight saponification was obtained. Undoubtedly there is always some saponification occurring, but this is not detected under the conditions outlined. In this connection, one must be cognizant of the fact that the flow rate of the lipid solution over the bed will depend on lipid concentration as well as on the UFA content and type of UFA present in the lipid under investigation. The amount and type of UFA being extracted onto the bed will influence the flow rate due to soap formation on the Celite surface. Long chain saturated acids have the greatest tendency to form soap films that retard flow. However, all of these factors can be readily compensated for by either lowering lipid concentration or following the column preparation technique described eariler, or both. It is usually the case when examining a lipid for the first time for UFA that some experimentation has to be done with regard to initial flow rate and lipid concentration.

Separation of the C_{10} through C_{18} saturated and the $C_{18:1}$, $C_{18:2}$ and $C_{18:3}$ UFA obtained under the specified conditions is depicted in Figure 2. Baseline separation between the $C_{18:0}$ and $C_{18:1}$ can be accomplished by decreasing the oven temperature from 195 to 185 C but this, of course, also increases the total analysis time and causes some broadening of the peaks.

Figures 3 and 4 are chromatograms obtained from the residues of the first and second passes of a sample of edible tallow and coconut fat, respectively. For the second pass of both lipids, note that the sensitivity of the instrument was 16 and 8 times greater than that used in the analysis of the first pass residue. The small peaks present on both second-pass chromatograms do not have retention times identical to any of the major acids present in their respective fats.

When all of the acids (C_{10} through $C_{18:3}$) are present in a fat or oil it is not possible, except under idealized conditions, to utilize an acid < C_{22} as an internal standard in order to obtain absolute concentrations of the UFA. The C_{22} acid, however, gives a broad, relatively flat peak that does not lend itself well to accurate measurement and also extends analysis time. The method, therefore, appears to be better suited to obtaining relative data based on peak heights and/or area measurements. Absolute data can, of course, be obtained by constructing standard curves for any or all of the UFAs on the chromatogram.

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